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Breast cancer requires estrogen for its initiation and maintenance before progressing into a more aggressive stage. We proposed to study the role of estrogen receptor (ER) target genes in the promotion of breast cancer. To achieve this goal we generated a regulable repressor KEDPK to directly turn-off the expression of ER target genes. The KEDPK contains an ER-DBD that recognizes the ERE elements of ER target genes, a KRAB repressive domain which can silence target genes when tethered to the promoter region and a mutated PR-LBD which responds only to exogenous ligand. The KEDPK shows dose-dependent inhibition of ER target genes in transient transfections. The inhibitory activity of KEDPK is under the control of its ligand RU486 and is specific to ER target genes. Currently we have devoted our effort to generate stable cell lines expressing KEDPK and determine its ability to suppress the endogenous ER target genes. The success of developing these repressors will allow us to study the role of ER target genes in breast cancer progression. Results obtained in these studies will be highly relevant to efforts in optimization of current hormone therapy and gene therapy for breast cancer.

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FOREWORD

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Significance

Breast cancer continues to be a prevalent disease among women in the United States. Breast cancer is promoted by activated ER before progressing to more aggressive stages. ER could be stimulated either by its ligand or alternatively by ligand-independent pathways. Some mutant form of ER activities are insensitive to antiestrogens and suspected to contribute to the growth of breast cancer from an estrogen-dependent to an estrogen- independent stage. It is well known that the activated form(s) of ER ultimately act on the regulatory region of its target genes to exert its biological function. Thus, we proposed to study of the role of ER target genes in breast cancer progression. The general approach is to construct a regulable repressor of ER target genes. Once the regulable repressor is expressed in tumor cells, the temporal repression of ER target genes can be closely regulated, rendering it feasible to define the role of ER target genes in mammary gland oncogenesis and in the recurrence of tumor growth in an estrogen-independent manner.

Specific aims

1) Generate a regulable system to shut-off estrogen receptor target genes by using several different constructs and vector systems. They will be used to optimize the system and improve its overall success.
2) Study the functional activity of the repressors on ER target genes in breast cancer cell lines. The expression of oncogenes normally amplified in breast cancer will be assessed in correlation with ER target gene expression.
3) Establish transgenic mouse lines expressing the regulable repressor. The validity of the regulable repressor will be evaluated in vivo by targeting its expression to breast epithelial cells. The effect of ER target genes on the transition to hormone-independent growth of breast cancers will be assessed by crossing the repressor expressing mice with breast cancer susceptible murine strains.

Research accomplishments

Generation of regulable repressor constructs

Regulable repressors were created by linking the KRAB domain (krupple-associated box repressive domain) to the ER DNA binding domain (DBD) and a truncated progesterone receptor ligand binding domain (PR-DBD). Upon binding to RU 486 the regulable repressor will dimerize and bind to estrogen responsive element (EREs) to suppress the ER target gene expression as depicted in Fig 1. Three constructs that contain the KRAB domain either at the N- (KEDP), C-terminal (EDPK) or at both ends (KEDPK) were made to assess which construct is the most potent repressor (Fig 2). The constructs were verified by sequence analysis (data not shown). All these constructs were placed under the control of the CMV promoter for functional test in transient transfection assay.

Regulable repressors inhibit ER dependent transcription in the presence of progesterone antagonist, RU486

To test the functional properties of each repressor, HeLa cells were co-transfected with the repressor and an $(ERE)_3$tata-Luc reporter plasmid together with a human ER expression vector. Luciferase activity in response to a saturating dose of E_2 (10^{-9} M) and RU486 (10^{-8} M) was measured in the absence or presence of the repressor plasmids. E_2 largely increased the ER dependent transcription in our transient transfection assay (data not shown). As shown in Fig 3A, ER dependent transcription was not affected by addition of RU486. The chimeric repressor KEDPK has no significant effect on the reporter gene expression in the absence of exogenous ligand, RU486. However, in the presence of RU486 (10 nM) KEDPK could effectively inhibit more than 80% of ER-mediated transcription, suggesting that KEDPK could compete effectively for the ERE binding site with wild-type ER to suppress the transcription of ER reporter. KEDP and EDPK could also inhibit ER induced transcription but was less potent than that of KEDPK (data not shown).

The specificity of repressor activity was assessed by co-transfection with a reporter plasmid containing glucocorticoid response element (GREtk-Luc) which contains a palindromic sequences similar to that of ERE. Since RU486 has been reported to have an antagonistic activity of glucocorticoid receptor (GR), a constitutively active form of GR (GR*) with a LBD truncation was used to exclude the effect of RU486 on GR activity. The truncated GR (GR*) stimulated GR reporter gene expression over 50 fold. The repressor has no effect on the GR* induced transcription either in the absence or in the presence of RU486 (Fig 3B). Thus, the repressor is a specific inhibitor of ER mediated transcription in transient transfection assays.

Dose-dependent inhibition of ER-mediated transcription by the inducible repressor

To further characterize repressor potency on ER-dependent transcription, different amounts of the KEDPK plasmid were co-transfected with the ER expression vector. Results shown in Fig 4A indicate that the inhibitory effect of the repressor on ER transcription was dose-dependent. A 50% reduction of ER-mediated transcription was observed when equal amounts of ER and repressor KEDPK plasmids were cotransfected. Maximum inhibition of the ER mediated transcription was observed when the repressor was in a four-fold excess of ER. Next, we assessed the effective dose of RU486 in inducing the repressor activity. As shown in Fig 4B, the repressor exhibited a RU486 dose-dependent regulation of suppressive activity. The maximum inhibition of ER mediated transcription is reached at concentration of 10 nM, which is below the concentration of RU486 required to antagonize progesterone or glucocorticoid receptors. This is the major concern in using RU486 in animal study.

KEDPK repression of ER-mediated transcription is independent of cellular and promoter context

ER contains two transactivation domains, AF1 and AF2, that operate in a cell and promoter-specific manner to mediated ER action. Tamoxifen, the most widely used agent in endocrine therapy of breast cancer, acts as a partial agonist of ER in a cell type specific manner. The partial agonist activity of tamoxifen has been proposed to relate to its ability to activate the AF1 of ER. To examine whether the repressor we developed is capable of inhibiting tamoxifen-activated transcription, the repressor plasmid and a $(ERE)_3$tata-Luc reporter were transfected together with an ER expression vector into

HepG2 (human heptocellular carcinoma) cells where the AF1 activity of ER was shown to be the major activation function. As shown in Fig 5, RU486 dependent repression of KEDPK has no significant effect on basal activity (lane 2). 4-hydroxy-tamoxifen (4OH-T) treatment of HepG2 cells resulted in an eleven-fold induction of ER mediated transcription (lane 5), which is about 20% of the response elicited by E_2 (lane 3). The repressor KEDPK inhibited the 4OH-T-induced ER activity in the presence of RU486 (lane 6 vs. lane 5) as efficiently as it inhibited E_2-induced activity (lane 4 vs. lane 3).

In addition, the efficacy of the repressor function was examined on a natural estrogen responsive promoter. In this case we chose the estrogen responsive complement factor 3 (C3) promoter which contains a putative ERE. E_2 was able to stimulate luciferase reporter expression from the natural C3 promoter (Fig 6). Transcription was effectively blocked (over 85% inhibition) in cells transfected with repressor after treatment with RU486 (10 nM). Taken together, these results indicated that repressor KEDPK could block the ER activity independent of cellular and promoter context.

Establishment of breast cancer cell lines stably expressing KEDPK repressor

The repressor KEDPK has been shown to specifically inhibit the ER reporter activity in the presence of RU486, with maximal effect of more than 80% inhibition in transient transfections. We next tried to generate stable cell lines expressing the repressor KEDPK in order to study the role of estrogen receptor target genes in breast cancer cell growth. The repressor KEDPK was subcloned into pcDNA3 expression vector that contains a neomycin-resistant marker. Breast cancer cell line MCF-7 have first been used for stable transfection. Neomycin resistant clones from both lines were selected in the presence of 300 μg/ml of neomycin. Eight clones have been selected so far and the effectiveness of KEDPK on ER target genes in these cell is under the test by transient transfections of $(ERE)_3$tata Luc reporter.

Ongoing experiments

It is imperative that the repressor KEDPK suppresses endogenous ER target genes in breast cancer cell lines. For this purpose, we will continue to generate more stable lines expressing KEDPK from both Estrogen dependent (MCF-7) and -independent (LTSD) breast cancer cell lines. The effectiveness of KEDPK on ER target genes in these stable cell lines will be screened first by transient transfection assay. The most promising cell lines will be selected for further characterization.

The ability of KEDPK to suppress endogenous ER response genes will evaluated by RNase Protection Assay (RPA) in comparison with its parental cell line. The pS2 gene will be used as an ER responsive gene marker to monitor the activity of the repressor KEDPK. The antisense probe of pS2 will be generated by subcloning RT-PCR amplified fragment corresponds to the coding region into pBS-KS plasmid (Stratagene). Since no antibodies available to detect specifically the regulable repressor KEDPK at protein level, an antisense probe corresponding to the KRAB region of KEDPK will be constructed to determine KEDPK mRNA in different stable cell lines.

Conclusion

As summarized above, we have achieved major progress toward our goals. We have successfully generated a regulable repressor that effectively and specifically inhibits the expression of ER target genes in transient transfection in the presence of RU486. More effort have been devoted to generate stable cell lines expressing KEDPK from both estrogen-dependent and -independent breast cancer cell lines. The ability of KEDPK to suppress the endogenous ER target genes will be determined once these stable cell lines are available.

Inducible Repressor for ER Target genes

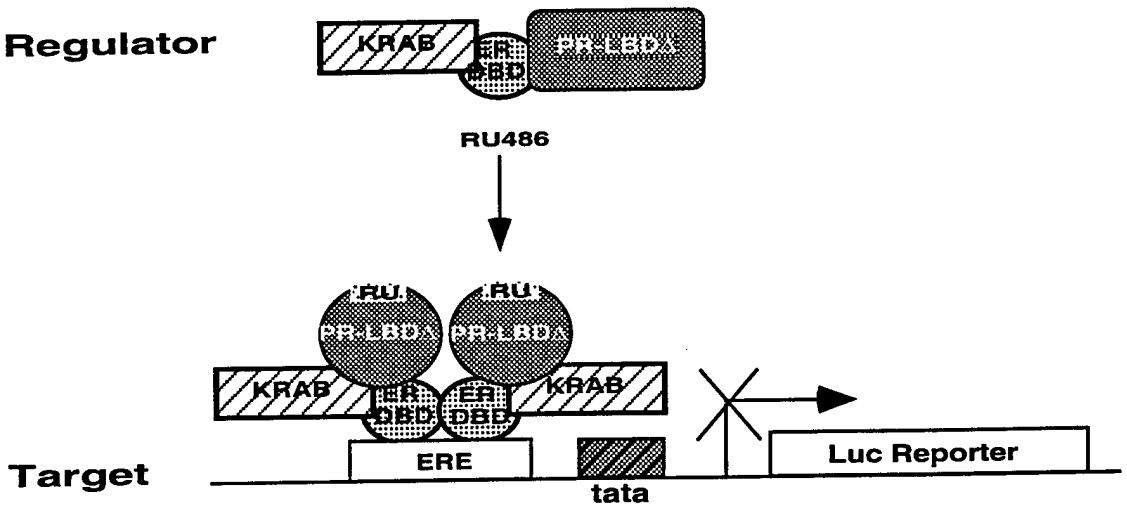

Figure 1

Diagram of Regulable Repressor Constructs

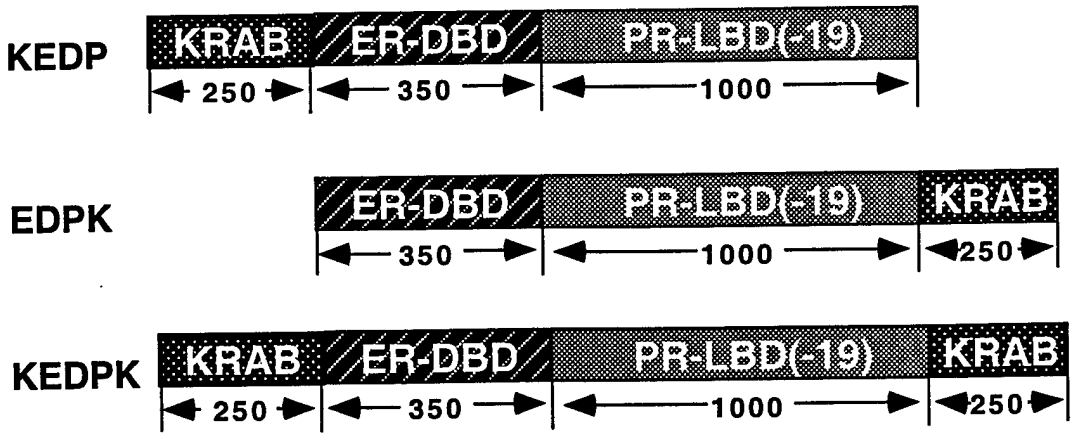

Figure 2

Specific Inhibition of ER-Dependent Activation by the Regulable Repressor, KEDPK

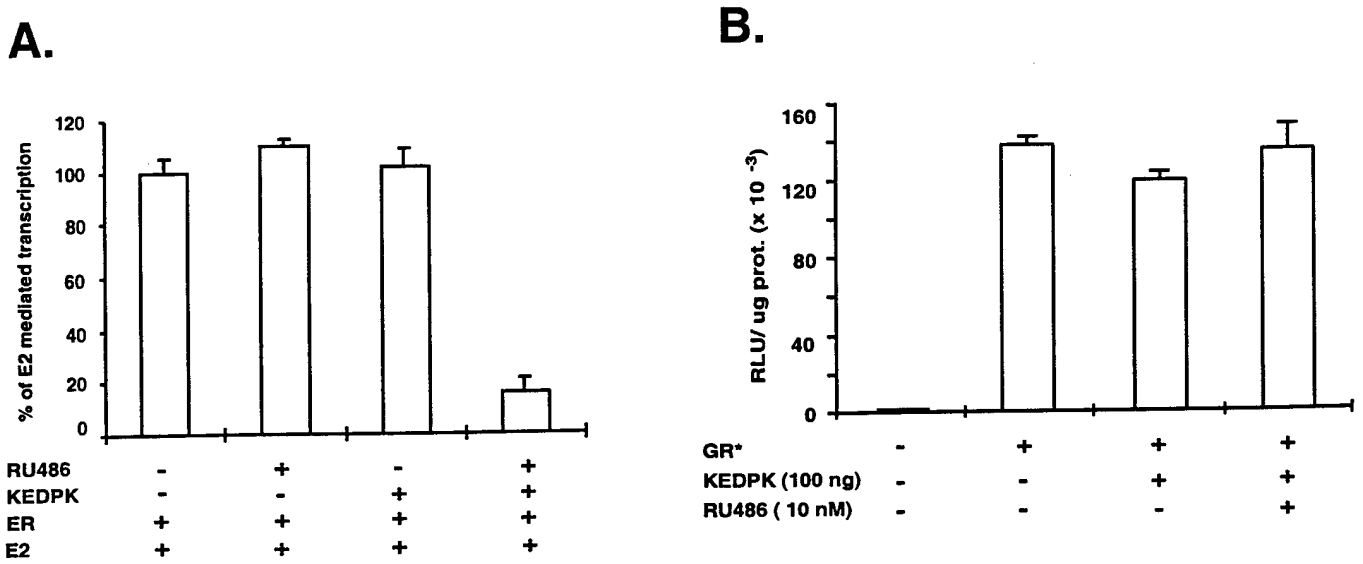

Figure 3

Inhibition of ER Activity by KEDPK in a Dose-Dependent Manner

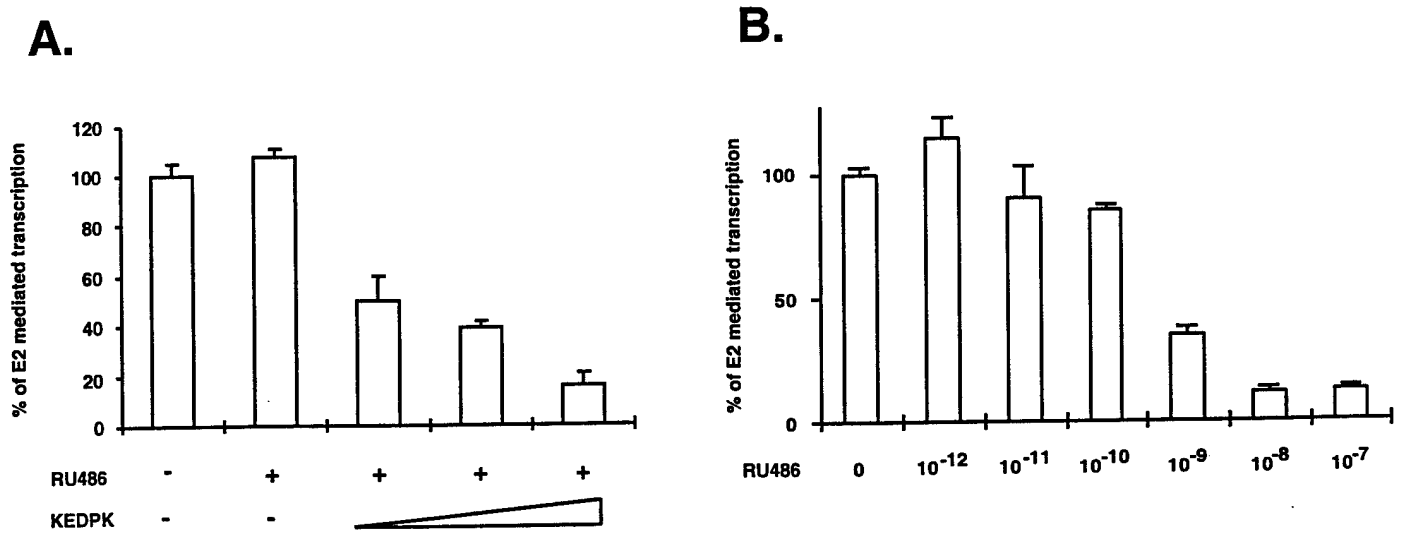

Figure 4

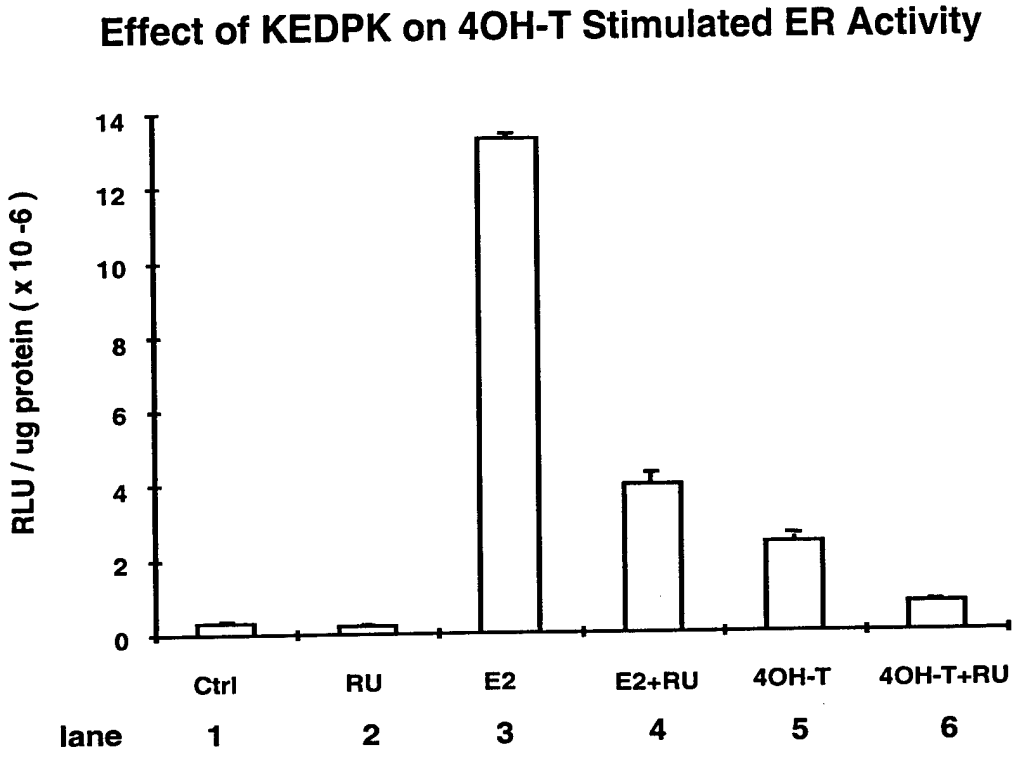

Figure 5

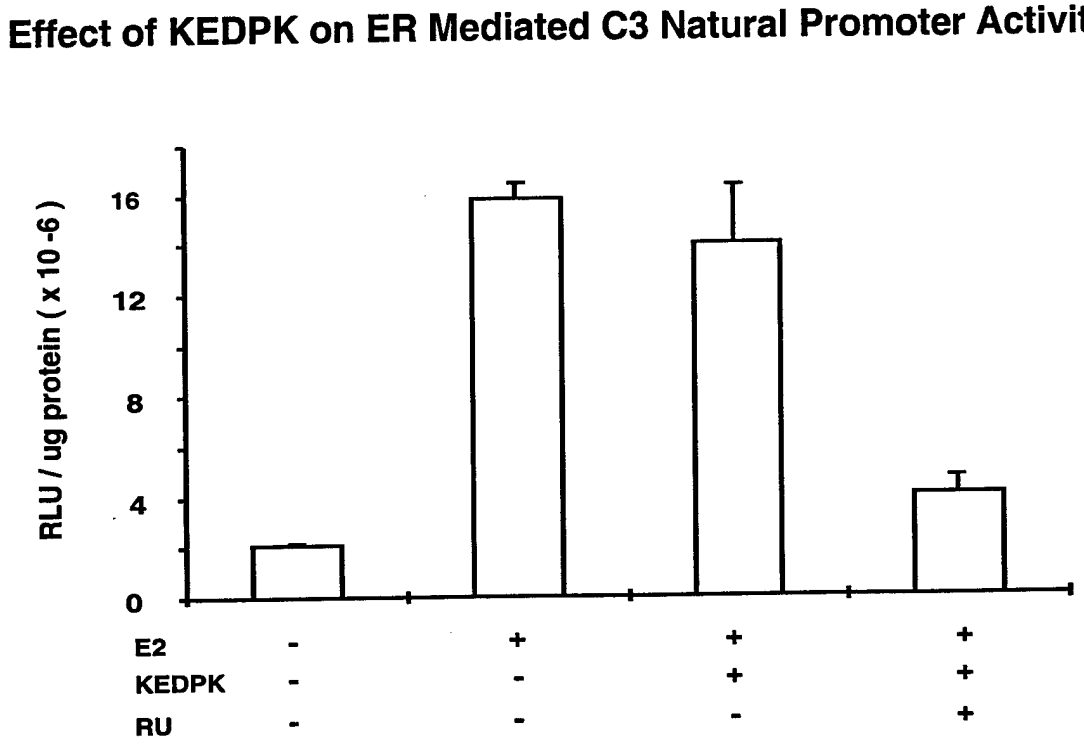

Figure 6

Legend

Figure 1. Model of inducible repressor system.

The regulable repressor contains a DNA-binding domain binding to estrogen response element (ERE), a transcription repression domain obtained from Krupple-Associated Box (KRAB), and a mutated PR ligand-binding domain that responds to antiprogestin, RU486. The regulable repressor constructed in this way can compete with wild-type ER for ERE binding and turn off all the ER target genes in breast cancer cells in the presence of RU486.

Figure 2. . Diagram of regulable repressor KEDP, EDPK and KEDPK constructs.

Figure 3. Specific inhibition of ER-dependent activation by the regulable repressor, KEDPK.

Panel A. An $(ERE)_3$tata-Luc reporter construct (100 ng) and a human ER expression plasmid (50 ng) was transfected along with or without regulable repressor, KEDPK (200 ng), into HeLa cells using lipofectin . After 6 hr of transfection, cells were washed and incubated in the presence of E_2 (1 nM) for an additional 24 hr with or without RU486 (10 nM), as indicated. The magnitude of ER activation by E_2 alone was set at 100%.

Panel B. HeLa cells were transfected with 50 ng of reporter GREtk-Luc, 50 ng of GR* (LBD truncation) with or without 100 ng regulable repressor, KEDPK. Luciferase activity was assayed 24 hrs after treatment with or without 100 nM RU486. Luciferase activity was normalized for protein.

A single experiment representative of at least two independent experiments is detailed above. The data shown indicates the mean ± SEM of quadruplicate estimations.

Figure 4. Inhibition of ER Activity by KEDPK in a dose-dependent manner.

Panel A. Cells were transfected with $(ERE)_3$tata-Luc reporter (100 ng), human ER expression plasmid (50 ng), and an increasing amount of KEDPK repressor construct (50, 100, 200 ng). Cells were treated with 10 nM 17ß-estradiol and 100 nM RU486 for 24 hrs as indicated.

Panel B. HeLa cells were transiently transfected with 50 ng of the $(ERE)_3$tata-Luc reporter, 50 ng of the human ER expression plasmid, and 200 ng of the repressor construct, KEDPK. Cultures were treated with 17ß-estradiol (10 nM) and different concentrations of RU486 for 24 hrs as indicated.

The magnitude of ER activation by E_2 alone was set at 100%. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean ± SEM of quadruplicate estimations.

Figure 5. The effect of KDEPK on 4-hydroxy-tamoxifen (4OH-T) stimulated ER activity.

The repressor plasmid (200ng) and $(ERE)_3$tata-Luc reporter (100 ng) were transfected together with an ER expression vector (100ng) into HepG2 (human heptocellular carcinoma) cells. Luciferase activity was normalized for protein. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean ± SEM of quadruplicate estimations.

Figure 6. The effect of KEDPK on ER mediated natural promoter activity of complement factor 3 (C3).

HeLa cells were transfected with C3-Luc promoter (100 ng), ER expression plasmid (100 ng), and KEDPK repressor construct (200ng). Cells were treated with 10 nM 17ß-estradiol and 100 nM RU486 for 24 hrs as indicated. Luciferase activity was normalized for protein. A single experiment representative of two independent experiments is detailed above. The data shown indicates the mean ± SEM of quadruplicate estimations.